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THE RELATION BETWEEN THE NUMBER OF BACTERIA AND ACID PRODUCTION IN THE FERMENTATION OF XYLOSE *

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The study of the rate of growth of bacteria and the extent of the changes in the composition of the nutrient medium which accompany growth has engaged the attention of bacteriologists for many years. The most important contributions to this problem may be divided along two lines: first, the relation between the number of bacteria and the amount of the end products at different times; second, involved mathematical equations and formulae to explain the rate of multiplication of bacteria in nutrient solutions. The usual method of investigation is to seed a small number of bacteria into a suitable medium under known conditions, and after given intervals of time to estimate the number by the plate method. The rate of development can be obtained for many kinds of bacteria by measuring their products, i. e., the extent of fermentation is assumed to be proportional to the number of bacteria that have developed.

This article is primarily concerned with the rate at which byproducts are formed and their relation to the number of bacteria. In order to study the mechanism of fermentation reactions, the rate of development of the micro-organism is of importance. At present, observations are not available which cover the whole period of growth from commencement of seeding to the period when growth ceases altogether, and by-products are no longer formed. It is true that there are ample observations available on the reproduction of bacteria and yeasts in the early stages of growth.

Nägeli and Schwendener[1] were perhaps the first to study the rate of growth of bacteria. They attempted to measure the reproduction of bacteria by the amount of products formed. For this purpose known dilutions of an impure

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[1] Das Mikroskop, 1877, p. 645.

culture of bacteria were prepared and the different dilutions used to inoculate a definite amount of culture medium. They found that the time required to produce a certain amount of by-products, e. g., acid from sugar, is proportional to the bacterial content.

Buchner, Longard, and Riedlin[2] used the Koch plate method in a study of the rate of growth of the cholera vibrio. From the results of counts made at the beginning and at the end of these tests, they calculated the time of generation. Their results indicate that cholera vibrio in a peptone sugar broth culture medium at 37 C., varies in its generation time from 19 to 40 minutes.

Mach and Portelle[3] in a study of alcoholic fermentation by yeast attempted to measure the growth of micro-organisms and the accumulation of their products. Their observations extended over the first 8 days of fermentation. During the first 2 or 3 days they obtained a rapid increase in the number of yeasts present, accompanied by a small production of alcohol and after 3 days the weight of the yeast remained constant, while the amount of alcohol rose rapidly. They determined the amount of yeast present by drying it at 100 C. and then weighing.

Rahn[4] carried out a number of experiments in the study of the influence of by-products on the reproduction of *B. flourescens.* He found that the substance which retards growth in a broth culture is removed when the culture is passed through a porcelain filter, treated with ether, or heated. His results show that this toxic substance is labile. In 1911 Rahn[5] determined the amount of acid formed in one hour by a single cell of Bact. lactis acidi. In the given time a single cell produced a quantity of acid approximately equivalent to its own weight. Rahn maintains that fermentation and growth are parallel in the life of an organism and that the apparent absence of fermentation in young cultures is misleading. The products are present, but in such small amounts that they cannot be measured.

Marshall and Farrand[6] in a study of the bacteria associated in the souring of milk, found that multiplication of bacteria and acid production are not always coincident. They concluded that certain types of organisms commonly found in milk accelerated the growth of the lactic acid bacteria and that other types retarded the production of lactic acid.

Berghaus[7] studied the relation between number of bacteria and ammonia production in a 1% peptone broth medium. Cultures of B. proteus, B. coli, B. typhosus, B. fecalis alcaligenes, B. prodigiosus, and cholera vibrio were used. He found that the bacteria multiply enormously within the first day or two, then decrease slowly. In general, the maximum number is found within 24 hours after inoculation. The formation of ammonia was not parallel to cell reproduction but reached a maximum many days after the bacteria showed a marked decline in number. Berghaus concluded that ammonia formation was not dependent on reproductive cells but might be produced by living, nonreproductive cells or by the substances given off from the dead cell.

Barber[8] studied the rate of reproduction of B. coli at different temperatures. By means of a capillary pipet he isolated a single organism and deter-

[2] Centralbl. Bakteriol., I, 1887, 2, p. 1.

[3] Landw. Vers-Station, 1892, 41, p. 261.

[4] Centralbl. Bacteriol., II, 1906, 16, pp. 417 and 609.

[5] Mich. Agric. Exper. Station Tech. Bull. 10, 1911.

[6] Centralbl. Bakteriol., II, 1908, 21, p. 7.

[7] Archiv. Hyg., 1908, 44, p. 3.

[8] J. Infect. Dis., 1908, 5, p. 379.

mined its rate of multiplication by direct observation under the microscope. He found no lag period in the growth of the organism when it was placed in a medium to which it was accustomed.

Lane-Claypon,[9] Penfold,[10] Ledingham and Penfold,[11] and Slator,[12] studied the mechanics of reproduction of micro-organisms in nutrient solutions and under the influence of certain factors. The mathematical equations governing the different phases of growth were discussed in detail.

According to Buchanan,[13] there are seven relatively distinct phases or periods in the life of a bacterial culture. Different equations are given for these seven phases in the development of a culture. Buchanan presents in this paper a review of the literature and a discussion of the mathematical equations.

A recent publication by Baker and his collaborators[14] compares the rate of bacterial multiplication with the rate of acid production in milk. They determined the number of bacteria present both by the plate method and by the direct microscopic count. Since lactic acid is one of the principal products formed by certain organisms in the souring of milk and in the fermentation of xylose, the data obtained by Baker and his co-workers are in some respects comparable to those which will be presented in this paper.

The experiments described were made in an attempt to determine the relations between the number of bacteria present and the rate of acid production during the fermentation of xylose. Experiments were planned to estimate the rate of growth of bacteria by means of plate counts and direct counts, and by the measurements of total acidity. The micro-organism chosen for this experiment was Lactobacillus pentoaceticus, an acetic and lactic acid producer, which occurs on plant tissue, silage, and manure. This micro-organism possesses many desirable points. It grows rapidly in a suitable medium, forms distinct, small, nonspreading colonies, is easily stained, and attacks the pentose sugar, xylose, rapidly, forming almost equal quantities of acetic and lactic acids. Some of the characters of this organism have been described in previous papers from this station.[15] Observations were also made to determine whether there was any change in the ratio between volatile and nonvolatile acids with increase in the

[9] J. Hyg. 1909, 9, p. 239.

[10] J. Hyg., 1914, 14, p. 215.

[11] J. Hyg., 1914, 14, p. 242.

[12] Biochem. J., 1913, 7, p. 197; Trans. Chem. Soc., 1916, 109, p. 2; J. Hyg., 1917, 16 p. 100; J. Soc. Chem. Ind., 1919, 38, p 391.

[13] J. Infect. Dis., 1918, 23, p. 109.

[14] N. Y. Agric. Exper. Station, Tech. Bull 74, 1919.

[15] Fred, E. B.; Peterson, W. H., and Davenport, Audrey, J. Biol. Chem., 1919, 39, p. 347; 1920, 41, p. 431; 42, p. 273.

age of the culture. Comparisons were made of the plate method of counting bacteria with the direct method of Breed.[16]

A 2% solution of xylose in a water extract of compressed yeast was prepared and sterilized for 30 minutes at 15 pounds' pressure. The culture medium was inoculated with pure culture of Lactobacillus pentoaceticus and incubated at 28 C.

TABLE 1

RELATION BETWEEN NUMBER OF BACTERIA AND ACID PRODUCTION FROM XYLOSE IN THE ABSENCE OF CALCIUM CARBONATE

Number	Hours Since Inoculation	Bacteria in 1 c c of Culture, Plate Counts	Total Titrable Acidity, 0.1 N in 100 c c of Culture, C c	Titrable Acidity by Bacteria, 0.1 N in 100 c c of Culture, C c
1	0	11,000,000	7.8	0.0
2	4	....	7.8	0.0
3	8	80,000,000	8.8	1.0
4	12	....	12.6	4.8
5	16	....	17.8	10.0
6	18	203,000,000	20.9	13.1
7	20	220,000,000	22.0	14.2
8	22	....	24.3	16.5
9	24	273,000,000	24.9	17.1
10	26	....	26.9	19.1
11	28	....	29.2	21.4
12	30	....	29.8	22.0
13	34	277,000,000	36.3	28.5
14	42	290,000,000	38.4	30.6
15	48	440,000,000	42.4	34.6
16	54	....	44.7	36.9
17	58	....	45.1	37.3
18	70	320,000,000	50.3	42.5
19	82	165,000,000	54.0	46.2
20	96	90,000,000	61.5	53.7
21	190	....	94.5	86.7

In the first experiment, the number of bacteria present was determined by the plate method, and in the second experiment both by the plate method and by the direct method of Breed. Because of the nature of the culture it was found necessary to employ a fixative for the direct microscopic preparation. Such a fixative was prepared by mixing equal portions of egg albumin and glycerol, and filtering through paper pulp. A very thin film of this solution was first dried on the slide, after which 0.01 c c of the culture solution was spread over 1 sq. cm. and then dried. With methylene blue the background stained a light blue and organisms a much darker blue. No attempt was made to count each individual cell in the microscopic preparations, if it occurred in a conglomerate, since groups of several organisms would give rise to a single colony on an agar plate. The figures obtained in the second experiment showed agreement between the two methods.

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[16] N. Y. Agric. Exper. Station, Tech. Bull 49, 1916.

The volatile acids were determined by distilling the culture with steam and titrating the distillate with 0.1 N barium hydroxid in the presence of phenolphthalein. The distillation was continued until less than 0.5 c c of barium hydroxid was required for the titration of 100 c c of distillate. After the removal of the volatile acids, the residue was evaporated to about 100 c c on a steam bath, and the nonvolatile acid extracted with ether by means of a Kutscher and Steudel extraction apparatus. This extraction was continued for about 72 hours. The flask containing the ether extract was then disconnected, 20 or 30 c c of water added and ether carefully distilled off. The solution was titrated while hot with 0.1 N barium hydroxid. Fred, Peterson and Davenport[15] found that the volatile acid obtained from the fermentation of xylose by Lactobacillus pentoaceticus is acetic, and the nonvolatile acid is lactic. Therefore, the only attempt made to identify the acids obtained was in the second experiment, in which the Duclaux constants of the volatile acid obtained from a 69-day old culture were compared with those obtained by Fred, Peterson, and Davenport from a 21-day old culture.

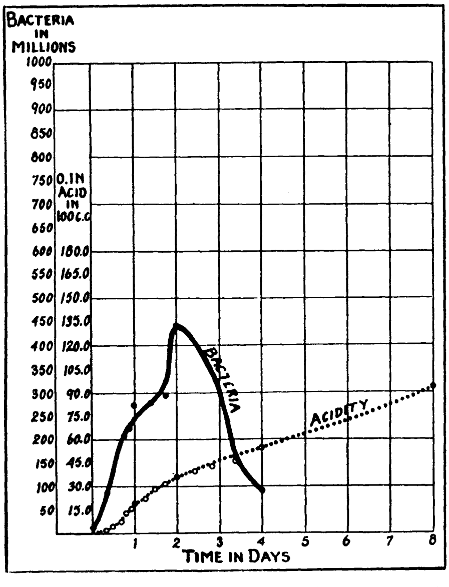

Chart 1.—Curves showing relation between total acidity and number of bacteria in the culture.

Experiment 1.—In this experiment 800 c c of a 2% xylose yeast water solution was inoculated with 10 c c of a 24-hour old liquid culture of Lactobacillus pentoaceticus, and incubated at 28 C. At the time of inoculation and at varying intervals thereafter, the number of bacteria per c c was determined. For this purpose, 1 c c of the solution was removed with a sterile pipet, proper dilutions made, and plates poured. In a similar manner 10 c c portions were removed at frequent intervals and titrated with 0.1 N barium hydroxid. These data are shown in table 1. According to these results, the period of most rapid multiplication occurred during the first 18 hours after inoculation, when the number increased from 11,000,000 to 203,000,000. From the eighteenth to the forty-second hour the bacteria multiplied rather slowly, and then made a rapid increase between the forty-second and forty-eighth hours, when the maximum number of 440,000,000

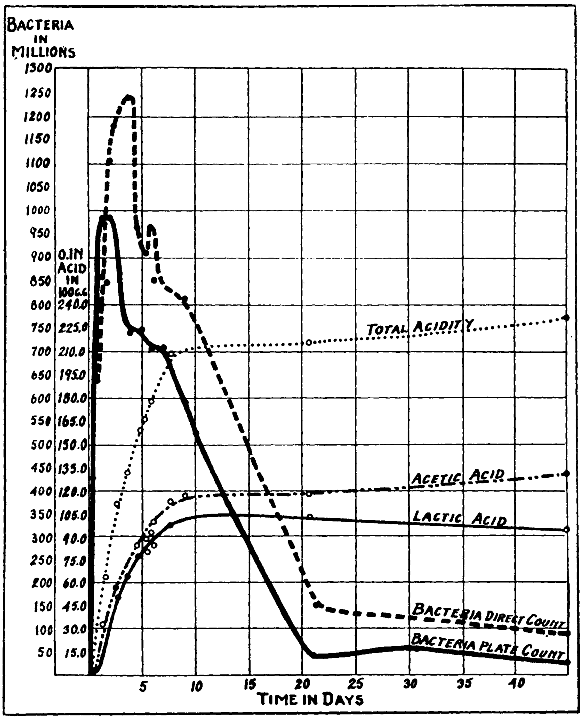

Chart 2.—Curves showing relation between number of bacteria and acidity.

organisms was reached. After this time the count gradually decreased until at the end of 96 hours only 90,000,000 bacteria were present.

The acidity rose most rapidly during that period in which the most rapid multiplication of bacteria took place. This is perhaps more clearly shown by chart I than by table 1. When the acidity reached a concentration of about 0.05 N the bacteria decreased in number very rapidly while the acidity increased slowly until, after 190 hours, the culture was very nearly a 0.1 N solution of acid.

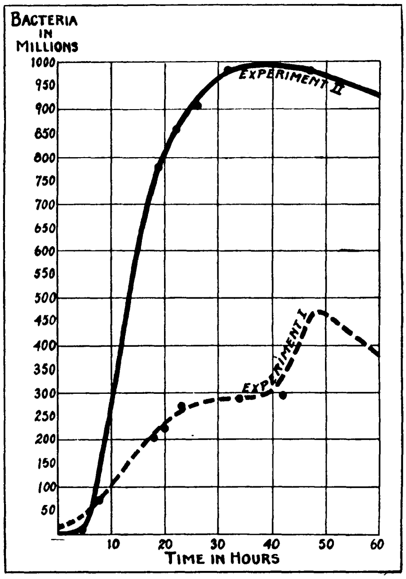

Chart 3.—Curves showing effect of calcium carbonate on number of bacteria. Exp. I, no $CaCO_3$ Exp. II with $CaCO_3$.

Experiment 2.—Three liters of a 2% xylose yeast-water solution were prepared in the same manner as in the previous experiment, except that after sterilization, an excess of calcium carbonate was added to the solution. It was then inoculated with 25 c c of a 24-hour culture of Lactobacillus pentoaceticus. Samples were removed from time to time by means of a sterile pipet for plate counts, and for the determination of volatile and nonvolatile acids. The results are shown numerically in table 2 and graphically in chart 2.

In this experiment a comparison was made between the plate method and Breed's direct method of counting bacteria. The direct count was usually made from a dilution of 1:100. When 1:100 c c of such a dilution was spread over 1 sq. cm. the microscopic fields could easily be counted. The fair agreement between the plate and direct counts is due to the fact that no attempt was made to count individual organisms. The higher counts obtained by the direct method are undoubtedly due to more than one factor, e. g., cells may stain which do not have the power of reproduction in agar. The direct count and the plate count show the same general increase and decrease. In the case of the plate count the maximum is reached at the end of about 30 hours and continues at this high level for about 18 hours when a gradual decline occurs. By the direct count the maximum number is reached somewhat later but attains a higher point. The presence of a neutralizing agent, calcium carbonate, permits an increased multiplication of the bacteria and a more nearly complete fermentation. If the rate of acid production in the second experiment be compared with that of the first, it apparently rises much more slowly. Perhaps this difference is more apparent than real. In the second experiment, calcium carbonate was present which prevented a direct titration of the acid. The method used in this second experi-

TABLE 2

RELATION BETWEEN NUMBER OF BACTERIA AND ACID PRODUCTION FROM XYLOSE IN THE PRESENCE OF CALCIUM CARBONATE

No.	Hours Since Inoculation	Bacteria in 1 c c of Culture		0.1 N Acid in 100 c c of Culture		
		Plate Count	Direct Count	Volatile, C c	Nonvolatile, C c	Total, C c
1	0	580,000	....	0.0	0.0	0.0
2	5	7,600,000	34,500,000			
3	15	....	430,000,000	0.59	0.30	0.89
4	19	775,000,000	639,000,000			
5	23	857,000,000				
6	27	907,000,000	....	11.15		
7	30	967,000,000	800,000,000			
8	39	....	842,000,000	32.93	30.70	63.63
9	43	850,000,000	929,000,000			
10	48	967,000,000	1,109,000,000			
11	64	....	1,188.000,000	57.25	52.30	109.55
12	88	877,000,000	1,245,000,000	69.40	63.40	132.80
13	91	740.000,000	1,099,000,000			
14	111	....	962,000,000	83.70	76.10	169.80
15	124	747,000,000	909,000,000	88.20	77.40	165.60
16	136	....	968,000,000	91.60	85.40	177.00
17	148	703,000,000	849,000,000	102.00		
18	166	707,000,000				
19	187	....	....	113.00	96.15	209.15
20	216	593.000,000	816,000.000	115.80		
21	244	533,000,000				
22	520	45,000,000	153,000,000	114.50	100.95	215.45
23	740	58,000,000	....	122.30		
24	1079	27,000,000	....	133.00	95.50	228.50
25	1652	12,500,000	56,600,000	146.90	93.10	240.00

ment no doubt does not measure accurately small amounts of acid. Moreover, in the presence of this basic substance, the longevity of the organisms is greater. An analysis for sugar was made on the culture at the conclusion of the experiment 69 days after inoculation, but only a trace of sugar was found. Fred, Peterson, and Davenport obtained a fermentation of about 80 to 90% of the sugar in the presence of calcium carbonate. The completeness of the fermentation in exper. 2 is perhaps due largely to the age of the culture. As in the previous experiment, the acidity increased rapidly for a short time after the number of bacteria had reached the maximum. A comparison between the increase in number of bacteria found in expers. 1 and 2 is shown by the curves of chart 3. The curve obtained in exper. 1 shows how strikingly the addition of calcium carbonate affects the increase in the number of bacteria in a xylose yeast-water culture medium.

TABLE 3

DISTILLING CONSTANTS OF THE VOLATILE ACIDS OBTAINED BY THE DUCLAUX METHOD

	Age of Culture, Days	10 C c	20 C c	30 C c	40 C c	50 C c	60 C c	70 C c	80 C c	90 C c	100 C c
Culture 118-8	21	7.5	15.6	23.7	32.2	41.3	50.8	61.1	72.2	84.7	100
Culture 118-8	69	7.7	15.9	24.4	33.2	42.3	51.9	62.2	73.3	85.7	100
Duclaux constant for acetic acid..	..	7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100
Difference.....	21	+0.1	+0.4	+0.3	+0.2	+0.4	+0.3	+0.5	+0.3	+0.3	
Difference.....	69	+0.3	+0.7	+1.0	+1.2	+1.4	+1.4	+1.6	+1.4	+1.3	

In the curves for lactic and acetic acid, chart 2, it will be seen that after 21 days the amount of acetic acid increased and the lactic acid decreased slightly. The last analyses for lactic and acetic acids are not indicated in the chart but are shown in table 2. This change in the ratio of acetic to lactic acid is due to a secondary fermentation which began after practically all of the xylose had been fermented. A similar destruction of lactic acid in old cultures of Lactobacillus pentoaceticus was noted by Peterson and Fred in the fermentation of glucose, fructose and mannitol. They have also demonstrated the ability of this organism to ferment solutions of sodium lactate with the formation of acetic acid and carbon dioxid. The disappearance of lactic acid from old cultures of other organisms was previously noted by Kayser,[17] Gayon and Dubourg,[18] and Duchacek.[19]

Nature of the Volatile Acid.—Because of the age of the culture, 69 days, it was thought that possibly volatile acids other than acetic might be formed. In order to test this point Duclaux analyses were made of the volatile acid residues. These constants compared well with those obtained by Fred, Peterson, and Davenport [15] (1919) in a 21-day old culture, as is shown in table 3. The results indicated that acetic acid was undoubtedly the only volatile acid produced during fermentation. The difference between the Duclaux constants obtained after 69 days and those obtained after 21 days was perhaps due to the difference in the original yeast-water, which usually contains a very small amount of volatile acid, the identity of which is unknown.

DISCUSSION

The results of these investigations and those obtained by others show that the fermenting capacity of a culture is determined by the number and age of the bacteria present. At first the rate of fermentation parallels the multiplication of the organisms. Perhaps this is due to the fact that the fermenting capacity of young cells is greater than that of old cells. There are fewer bacteria in an old culture and from a comparison of the rate of acid formed in a given time it appears that the cells have a diminished fermenting capacity. These organisms may form compounds other than the main products of fermentation, which are toxic to their growth.

Slator [12] found that the low oxygen tension brought about by the carbon dioxid produced by yeasts was injurious to the cells before the concentration of alcohol became high enough to be harmful. Perhaps a somewhat similar condition is found in the case of Lactobacillus pentoaceticus but with a substance other than CO_2.

Rubner [20] says that growth of bacteria is comparable to that noted in animals; namely, that reproduction is a characteristic of young cells which may be followed by a long period of active existence in which the cell continues to function. Growth and life after growth are but stages in the general functions of a cell. As shown by the yeasts, many micro-organisms possess the power of bringing about metabolic changes without reproduction. It must be remembered that by means of enzymes given off from the cell, bacteria can bring about changes that result in a preparation of the food for the cell. Thus fermentation may be concerned with autolytic processes, brought about by the cells that are unable to reproduce. That the temperature at which optimum reproduction occurs is not necessarily the optimum temperature for the production of byproducts has been clearly demonstrated by a number of investigators. The most favorable temperature for fermentation by Lactobacillus pentoaceticus is 28 C. Whether this temperature is also the most favorable for reproduction has not been determined.

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[17] Ann. de l'Inst., Pasteur, 1894, 8, p. 737.

[18] Ann. de l'Inst. Pasteur, 1894, 8, p. 108; 1901, 15, p. 527.

[19] Centralbl. Bakteriol., I, 1904, 37, p. 326.

[20] Archiv. Hyg., 1906, 57, p. 161.

It has been stated by others that fermentation does not occur until some time after the inoculation of a culture and that the length of time before fermentation begins is dependent on the temperature, age, and activity of the cells, adaptability of the organism to its new environment, and size of the inoculum. The fact that so few organisms are present as to make a detection of their fermentation products impossible, does not necessarily mean that no fermentation has taken place. Since fermentation may be coincident with growth and reproduction, or may occur without reproduction, it seems safe to conclude that fermentation may commence as soon as a solution is inoculated with an organism.

CONCLUSIONS

The rate of acid production in the fermentation of xylose by Lactobacillus pentoaceticus is most rapid during the period of maximum growth of the bacteria. A decline in the rate of growth is accompanied by a decrease in acid production although a slow acid formation is noted for many days.

In the first stages of growth, the curves of multiplication of bacteria and of acid formation are almost parallel although growth precedes the formation of acid in measurable quantities. The maximum number of bacteria occurs in the early stages of fermentation, usually within 48 hours after inoculation, while the maximum acidity is not noted for several days. In a xylose yeast-water medium the bacteria multiply more rapidly and reach a higher number if calcium carbonate is present.

In old xylose cultures of Lactobacillus pentoaceticus, the ratio between lactic and acetic acids may change. This is probably due to a secondary fermentation of the lactic to acetic acid.

Apparently, acetic acid is the only volatile acid produced in the fermentation of xylose by Lactobacillus pentoaceticus.

Direct count and plate count of the total number of bacteria give the same general results.